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(54) Enzyme electrode membrane and method of making same

(57) An enzyme electrode membrane is disclosed comprising an immobilised enzyme containing layer 2 supported on a polymeric base comprising a layer 1 of alkoxy-substituted polyamide, preferably a spun cast layer of methoxynylon with a thickness in the range 0.5 to 1 micron. Additional base layers may be present, e.g. of spun cast cellulose, cellulose proprionate or spun cast polyacrylic acid, to prevent potentially interfering species in the test sample from reaching the electrode surface. If desired and indeed preferably the surface of the membrane is protected by a porous layer, e.g. of polycarbonate 3.

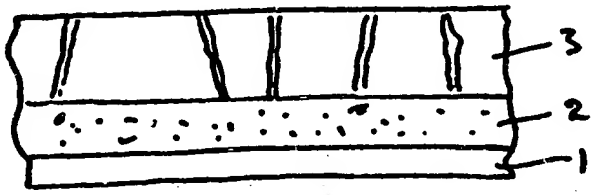

Fig 1

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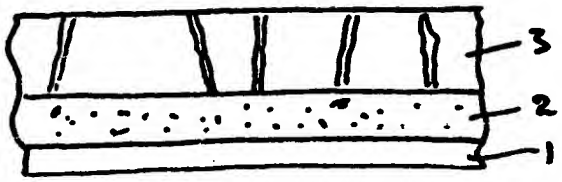

Fig 1

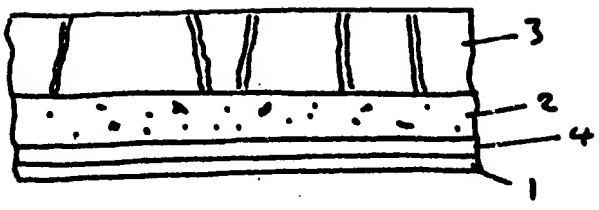

Fig. 2.

SPECIFICATION

Enzyme electrode membrane and method of making same

This invention relates to enzyme electrode membranes and a method of making same.

Polarographic cell systems employing an enzyme electrode membrane, i.e. a membrane having an enzyme immobilised therein or thereon, have been used extensively in recent years for the detection and measurement of various substances, particularly substances present in relatively small amounts in medical or clinical samples. A particular example is a glucose membrane comprising immobilised glucose oxidase and which may be used to measure blood sugar levels via the reaction:

$$\text{glucose} + O_2 \xrightarrow[\text{oxidase}]{\text{glucose}} \text{gluconic acid} + H_2O_2$$

Although glucose itself is not polarographically active, both gluconic acid and hydrogen peroxide are, and are therefore detectable and measurable as a measure of the glucose content of the original sample. Other substances which can be detected and measured using similar techniques include uric acid, urea, cholesterol and some drug molecules.

Enzyme electrode membranes devised for this purpose generally comprise a sandwich-type construction i.e. comprising a layer of enzyme immobilised between two opposing porous films or layers, although some two-layer constructions have been proposed consisting simply of the porous backing layer or film and the immobilised enzyme. One of the first proposals in this field was for an enzyme electrode membrane comprising glucose oxidase immobilised between layers of cuprammonium regenerated cellulose (Cuprophane): Annals of the New York Academy of Science, Volume 102 pages 29–45 (1962). The Cuprophane layers, in that case, were permeable not only to the substance to be measured, i.e. glucose, but also to the products of the enzymic reaction, viz. gluconic acid and hydrogen peroxide, either of which could be detected, for example, by a pH electrode in the case of gluconic acid, or at a platinum anode in the case of hydrogen peroxide, see for example, US-A-3,539,455.

Since that time enzyme electrode membranes have become much more sophisticated, both in design and in the range of materials used, with the object always of improving sensitivity and cutting out interference from other interfering species, i.e. species which interfere directly or indirectly with the final measurement, and which may be present either in the original sample, or produced as a by-product of the enzymic reaction.

The following is a representative selection of prior art disclosures:

GB-A-1,554,292. Yellow Springs Instruments Co. Inc. (Newman). This discloses an immobilized enzyme membrane for the polarographic determination of a hydrogen peroxide producing substance, e.g. glucose, comprising a first layer of silicone rubber, polymethyl methacrylate or cellulose acetate having a thickness of less than 2 microns and permeable to hydrogen peroxide but substantially impermeable to substances of higher molecular weight, a second layer, e.g. of porous polycarbonate, having a thickness of 1 to 20 microns, permeable to hydrogen peroxide, and the substance to be measured, i.e. glucose, but allegedly impermeable to other substances of higher molecular weight, and an ahdesive layer bonding the two, said adhesive layer consisting of or comprising the enzyme.

EP-A-0 079 502. Miles Laboratories Inc. (Oberhardt). This discloses a multilayer enzyme electrode membrane comprising a first relatively dense layer e.g. of cellulose acetate, on which are deposited an alternating array of porous polymer and immobilised enzyme layers, e.g. alternating layers of porous cellulose acetate deposited by a phase inversion technique, i.e. casting from a solution containing both a solvent and a non-solvent for the cellulose acetate, and layers of glutaraldehyde cross-linked glucose oxidase. The invention overcomes the problems allegedly associated with a single layer of cross-linked enzyme, by providing a more homogeneous distribution of enzyme throughout the membrane. Multiple layers of different porosity may be used.

EP-A-0 080 502. Miles Laboratories Inc (D'Orazio). This discloses an essentially two layer enzyme electrode consisting of a relatively dense layer, e.g. of cellulose acetate, and an overlying relatively porous layer, also of cellulose acetate, formed by phase inversion casting, and comprising the enzyme dispersed throughout the porous layer.

In both the Yellow Springs patent and the published applications by Miles, traditional casting techniques are used for both the base and subsequent layers, mainly solvent casting of the film onto a strippable substrate. Individual film thicknesses generally range from 1 to 20 microns, with total membrane thicknesses in the range 40 to 100 microns, although suggestions are made in the Yellow Springs patent for sub-micron size support layers, i.e. the cellulose acetate, silicone rubber or polymethyl methacrylate layer, with suggested thicknesses in the range 0.5 to 1.0 micron, and an overall membrane thickness of less than 10 microns.

To be effective, enzyme electrode membranes for medical and clinical diagnostic purposes must possess a number of characteristics:

(1) they must be permeable to hydrogen peroxide or other molecular species to be

measured;

(2) they must be impermeable to ascorbic acid, paracetamol and other molecular species which might otherwise interfere with the polarographic detection and measurement of the hydrogen peroxide produced by the reaction between the enzyme and its substrate, i.e. the substance to be detected or determined;

(3) they should be impermeable to molecular species which interfere with the enzymatic reaction;

(4) they should give a rapid linear response;

(5) they should be strong enough to withstand cleaning;

(6) they should not delaminate on soaking;

(7) they should retain their enzymatic activity and response times over long periods.

Existing enzyme electrode membranes meet some but not necessarily all those requirements, and to a greater or lesser extent, leaving room still for the development of improved enzyme electrode membranes.

One of the particular problems in the design of new and improved enzyme electrode membranes is in the construction of the base layer of the membrane in view of the often conflicting requirements of physical strength, permeability to hydrogen peroxide, impermeability to other interfering species, bondability etc. etc., all of which are not necessarily found in one polymer.

In accordance with the present invention we have found that methoxynylon, and other lower alkoxy substituted polyamides, surprisingly, have excellent properties as the material for the base of an enzyme electrode membrane. Polyamides, in general, have low permeability, of the order of 20 to 30 times less than that of cellulose acetate, due to their polar nature, and could not be expected to provide adequate transportation of hydrogen peroxide through the membrane for the purposes of determination at the electrode. Alkoxy substituted polyamides, particularly methoxynylon, are, however, highly hydrophilic, with a high uptake of water resulting in a hydrogel structure through which hydrogen peroxide and other molecules can readily diffuse. Alkoxy polyamides also show good strength, and good adhesion both to the immobilised enzyme layer, and to the outer protective, microporous layer if used, thus giving rise to robust membranes which are easy to clean, strong, resistant to delamination and of good sensitivity.

In one aspect, therefore, this invention provides an enzyme electrode membrane comprising a layer of immobilised enzyme bonded to the surface of a polymeric base, wherein the base consists of or comprises a layer of an alkoxy-substituted polyamide, preferably methoxynylon. If desired, the surface of the immobilised enzyme layer may further be protected by a microporous, ultra-filtration membrane of film, preferably a film of microporous polycarbonate sold under the trade name NUCLEPORE.

The polymeric base to the enzyme electrode membrane of this invention may have a thickness ranging from 0.5 to 20 microns or more depending on physical strength and other requirements, but in general terms, the thinner the base, the better, i.e. quicker, the response times, and the better the sensitivity of the membrane as a whole.

If desired, the base may comprise one or more sub-layers, in addition to the alkoxy polyamide layer, to prevent the passage of undesirable molecular species through to the electrode, and which might otherwise interfere with the polarographic determination of the hydrogen peroxide, or other low molecular weight species to be determined, depending on the precise nature of the enzyme used, and the substance, e.g. glucose or cholesterol, to be determined. For example, by including a sub-layer of cellulose propionate, for example, or a polyelectrolyte such as polyacrylic acid, membranes can be obtained which are impermeable, respectively, to paracetamol and ascorbic acid, which are known to adversely affect blood sugar determinations in patients taking paracetamol, or other analgesics, or vitamin C, prior to measurement of their blood sugar levels.

The substituted polyamides used in this inventin are, as indicated, alkoxy-substituted polyamides preferably alkoxy-substituted polyamides having 1 to 4 carbon atoms in the alkoxy substituent. The nylon base polymer is preferably nylon 66, but other polyamides may be used e.g. nylon 6, nylon 610 etc. Preferred are methoxynylons sold under the Registered Trade Mark ELVAMIDE (DuPont).

For use in the present invention, methoxynylon and other alkoxy polyamides can be formed into thin films by a variety of techniques, e.g. roller coating, doctor blade, etc., but preferably the thin films are formed by spin casting a solution of the polymer in a suitable solvent. In this way ultra-thin films can be obtained having uniform thicknesses ranging from a few microns, e.g. 1 to 5 microns, down to as low as 0.5 or even 0.1 micron.

In performing the above method, the steps can be performed in any order. For example, and most preferably, the base of the membrane is first formed by spin casting the methoxynylon and other base layers, if used. Once the base has been formed the immobilised enzyme containing layer is formed in situ on the base in known manner, for example, by applying to the surface of the base a paste comprising the enzyme in admixture with a cross-linking agent such as glutaraldehyde or hexamethylene diisocyanate, thereby to cross-link the enzyme *in situ* on the surface of the base, or by applying to the base a gelatin solution to which the enzyme has been added,

or by the technique disclosed in EP-A-0 080 502, i.e. phase inversion casting of a solution containing the enzyme in admixture with a suitable polymeric matrix matrial, e.g. cellulose acetate. As the final step, the immobilised enzyme layer may or may not be protected by the optional upper, e.g. microporous, surface film or layer.

Alternatively, the membrane may be made in reverse order, i.e. by first forming an immobilised enzyme layer, with or without the microporous protective layer, and spin casting the base layer directly onto the immobilised enzyme layer.

Sections through typical membrane constructions according to the invention are shown in the accompanying drawings. According to the first embodiment, Fig. 1, the membrane is composed of a plurality of ultra-thin, preferably spun cast, base layers 1 of methoxynylon, an immobilised enzyme layer 2, and optionally the microporous surface layer 3.

If desired, the base may also comprise an additional sub-layer 4, for example, of cellulose propionate, which may be incorporated into the base to provide substantial impermeability to paracetamol, or a polyelectrolyte film, e.g. of polyacrylic acid, which can be incorporated to provide impermeability to ascorbic acid.

As the immobilised enzyme there may be used any enzyme capable of reacting with the substance (enzyme substrate) to be determined to produce a polarographically detectable species, e.g. hydrogen peroxide. Typical examples are glucose oxidase for the determination of glucose, cholesterol oxidase for the determination of cholesterol and urease for the determination of urea, and uric acid. The enzyme may be immobilised in the membrane by various techniques already mentioned.

Finally the immobilised enzyme layer may be further protected by outer or upper layers, e.g. a microporous or ultra-filtration membrane, permeable at least by the substance to be determined, and preferably substantially impermeable to larger molecular species which may be present in the sample and which may otherwise interfere with the enzymatic reation and/or the subsequent polarographic measurement. Such protective membranes and techniques are known and need not be further described. The preferred microporous or ultra-filtration membrane material used to protect the enzyme layer in the membrane of this invention is a microporous polycarbonate, e.g. the material sold under the trade name NUCLEPORE.

Membranes according to this invention are illustrated by the following examples.

EXAMPLE 1

Glucose Biosensor Membrane with Paracetamol Rejection

A membrane suitable for a glucose biosensor showing low interference to the drug paracetamol, (present in the blood of persons taking analgesics) is manufactured in the following way.

A polymer solution of 5% methoxy nylon, ELVAMIDE 3061, is made by dissolving 5 grams of the polymer in 47.5 grams of methanol "ANALAR" and 47.5 grans of chloroform "ANALAR". When the dissolution is complete, the solution is ready for use.

Additionally, a solution of cellulose propionate is also made by dissolving 10 grams of cellulose propionate in 90 grams of acetone.

Thirdly an enzyme solution is made by dissolving 0.1 grams of glucose oxidase in 8 mls of phosphate buffer (pH 7) and when the enzyme has dissolved completely a 2 ml solution of glutaraldehyde (25% aqueous) is injected into the solution and quickly stirred in.

A piece of polyethylene terephthalate sheet, MELINEX, is placed on the vacuum chuck of a vacuum photoresist spinner and cleaned by spinning 5 mls of filtered acetone over the surface; during this procedure the spinning speed is adjusted to 1400 rpm. A small amount of the 5% ELVAMIDE solution, 5 ml, is then placed in the centre of the MELINX sheet and spun at 1400 rpm for 1 minute.

Next 5 ml of the cellulose propionate solution is placed on the spun film of methoxynylon, still on the chuck, and the chuck respun at 8000 rpm for 1 minute. As a result a thin film of cellulose propionate is spun on top of the methoxynylon film.

1 ml of the freshly prepared enzyme solution is placed on the centre of the spun cellulose propionate film and spun for 5 seconds at 1400 rpm.

Then a disc of polycarbonate Nuclepore membrane (0.03 μm pore size) is brought into contact with the enzyme layer surface by being applied on a small hand roller. The membrane is found to stick well to the surface. It is then removed intact from the vacuum chuck and placed between glass plates under pressure after having a MELINEX sheet placed on top. The assemblage is placed in an oven at 45°C for 30 minutes to ensure chemical reaction and adhesion.

When the heating period is complete, the assembly is removed from the oven, allowed to cool and then separated from the surfaces of the glass. The membrane is then peeled off the surface of the MELINEX and the O-rings attached adhesively to the base side. The individual O-rings are then cut into individual pieces and packed in dry cool conditions. Such a membrane is suitable for use in an electrochemical sensor and can be used to measure blood glucose concentration in the physiological range, when used with a calibration solution.

It is found that the membrane shows linear correlation with blood glucose concentrations,

but negligible response to normal physiological levels of paracetamol. The membrane is therefore very suitable for measuring blood sugar levels of patients who hve taken paracetamol tablets before measurement.

EXAMPLE 2

Glucose Membrane with Ascorbate Rejection

A membrane suitable for measuring blood glucose using a glucose biosensor can be manufactured in the following way. Such a membrane has the special characteristic that ascorbate ions present due to the injection of vitamin C tablets are not able to penetrate the membrane and give false readings at the biosensor electrode surface.

The means of manufacture of the membrane is similar to that of Example 1, except that in place of the cellulose propionate solution there is used a solution of 5% polyacrylic acid in water neutralised to pH 7 with sodium hydroxide solution which is spun at 5000 rpm on top of the methoxynylon layer.

Due to the exclusion effect, DONNAN, such a membrane gives a linear response to blood glucose concentrations but is not affected by high concentrations of the ascorbate ion found in patients taking large amounts of vitamin C tablets.

EXAMPLE 3

Glucose Membrane

A membrane suitable for a glucose biosensor is manufactured in the following way.

A polymer solution of 5% methoxynylon, ELVAMIDE 8061, is made by dissolving 5 grams of the polymer in 47.5 grams of methanol and 47.5 grams of chloroform. When the dissolution is complete, the solution is ready for use.

An enzyme solution is also made for application to the top of the base layer. This is made by dissovling 0.1 grams of glucose oxidase in 8 mls of phosphate buffer (pH 7). When the enzyme has dissolved completely a 2 ml solution of glutaraldehyde (25% aqueous) is injected into the solution and quickly stirred in.

A piece of polyethylene terephthalate sheet, (Registered Trade Mark MELINEX: ICI) is placed on the vacuum chuck of a vacuum photoresist spinner and cleaned by spinning 5 mls of filtered acetone over the surface. During the procedure the spinning speed is adjusted to 1400 rpm. A small amount of the 5% ELVAMIDE solution, 5 mls, is placed in the centre of the MELINEX sheet and spun at 1400 rpm for 1 minute to form a thin film. After this a small amount of the enzyme solution, 1 ml, is placed on the centre of the methoxynylon film and spun for 5 seconds at the same low speed.

Then a disc of porous polycarbonate film (NUCLEPORE, 0.03 μm pore size) is brought into contact with the enzyme layer surface by being applied on a small hand roller. The membrane is found to stick well to the surface. The membrane is then removed intact from the vacuum chuck and placed between glass plates under pressure after having a MELINEX sheet placed on top. The assemblage is placed in an oven at 45°C for 30 minutes to ensure chemical reaction and adhesion.

When the heating period is complete the assembly is removed from the oven, allowed to cool and then separated from the glass plates. The membrane is then peeled off the surface of the MELINEX and the O-rings attached adhesively to the base side. The individual O-rings are then cut into individual pieces and packed in dry cool conditions. Such a membrane is suitable for use in an electrochemical sensor and can be used to measure blood glucose concentration in the physiological range, when used with a calibration solution.

EXAMPLE 4

Cholesterol Membrane

Example 3 is repeated but using an enzyme solution comprising 0.1 g cholesterol oxidase, 2 ml of 25% aqueous glutaraldehyde solution and 8 ml phosphate buffer solution pH 7 in place of the glucose oxidase solution. 2 ml of this cholesterol oxidase solution is spun cast onto the methoxynylon film at 1400 rpm for 5 seconds. The enzyme layer is then covered with a disc of NUCLEPORE, and processed as before.

CLAIMS

1. An enzyme electrode membrane comprising an immobilised enzyme-containing layer supported upon a polymeric base layer permeable to hydrogen peroxide or other low molecular weight polarographically detectable species produced by the enzymatic reaction of said enzyme and the substance to be determined, wherein the polymeric base layer consists of or comprises a film of an alkoxy-substituted polyamide.

2. An enzyme electrode membrane according to claim 1, wherein said alkoxy-substituted polyamide is a methoxynylon.

3. An enzyme electrode membrane according to claim 1 or 2, wherein said polymeric base layer comprises a spun cast film of alkoxy-substituted polyamide having a thickness in the range 0.5 to 1 micron.

4. An enzyme electrode membrane according to claim 1, 2 or 3, wherein the polymeric base additionally comprises a spun cast film of cellulose propionate and/or a spun cast film of polyacrylic acid.

5. An enzyme electrode membrane according to any one of claims 1–4, wherein the immobilised enzyme layer comprises, as said enzyme, a glucose oxidase or cholesterol oxidase.

6. An enzyme electrode membrane according to any one of claims 1–5, wherein the surface of the immobilised enzyme containing layer is further protected by a microporous ultra-filtration membrane.

7. An enzyme electrode membrane according to claim 6, wherein said microporous ultra-filtration membrane comprises a layer of microporous polycarbonate.

8. An enzyme electrode membrane according to claim 1 for use in the determination of blood sugar levels consisting essentially of a spun cast film of methoxynylon, an immobilised glucose oxidase-containing layer bonded to the methoxynylon layer, and a microporous polycarbonate film overlying the immobilised enzyme layer.

9. An enzyme electrode membrane according to claim 1 for use in the determination of blood cholesterol levels consisting essentially of a spun cast film of methoxynylon, an immobilised cholesterol oxidase-containing layer bonded to the methoxynylon layer, and a microporous polycarbonate film overlying the immobilised enzyme layer.

10. An enzyme electrode biosensor incorporating an enzyme electrode membrane as claimed in any one of the preceding claims.

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